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(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventor: DE BOER, Mark; Elisa van Calcarstraat 30, NL-1963 DG Heemskerk (NL).

(74) Agents: SAVEREIDE, Paul, B. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).

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(57) Abstract

Methods for preventing or treating an antibody-mediated disease in patient are presented, the methods comprising administration of a humanized monoclonal antibody or fragment thereof that is capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell. Humanized monoclonal antibodies and fragments useful in these methods are also presented.

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HUMANIZED ANTI-CD40 MONOCLONAL ANTIBODIES AND FRAGMENTS CAPABLE OF BLOCKING B CELL ACTIVATION

FIELD OF THE INVENTION

This invention relates to antibodies and methods for treating diseases of the immune system. In particular, this invention relates to humanized anti-CD40 antibodies and methods of preventing or treating antibody-mediated diseases such as IgE-mediated disease (allergies) and autoimmune diseases including systemic lupus erythematosus (SL), primary biliary cirrhosis (PC), idiopathic thrombocytopenic purpura (ITP), and rheumatoid arthritis (RA).

BACKGROUND OF THE INVENTION

I. B-Cell Activation

B cells play an important role during the normal *in vivo* immune response. A foreign antigen will bind to surface immunoglobulins on specific B cells, triggering a chain of events including endocytosis, processing, presentation of processed peptides on MHC-class II molecules, and up-regulation of the B7 antigen on the B-cell surface. A specific T cell then binds to the B cell via T-cell receptor (TCR) recognition of processed antigen presented on the MHC-class II molecule. Stimulation through the TCR begins to activate the T cell and initiates T-cell cytokine production. Interaction between the CD28 antigen on T cells and the B7 antigen on B cells can provide a second signal further activating the T cell, resulting in high level cytokine secretion. Additionally, the CD40 ligand, which is not expressed on resting human T cells, is up-regulated on the T-cell surface when the above-mentioned signals are received. The B cell is then stimulated by The CD40 ligand through the CD40 antigen on the B-cell surface, and also by soluble cytokines, causing the B cell to mature into a plasma cell secreting high levels of soluble immunoglobulin.

II. The EL4B5 Cell Line

A few years ago, Zubler *et al.*, J. Immunol. (1985) 134:3662, observed that a mutant subclone of the mouse thymoma EL-4 line, known as EL4B5, could strongly stimulate B cells of both murine and human origin to proliferate and differentiate into immunoglobulin-secreting plasma cells *in vitro*. This activation was found to be antigen-independent and not MHC restricted. For optimal stimulation of human B cells, the presence of supernatant from activated

human T cells was needed, but a B-cell response also occurred when EL4B5 cells were preactivated with phorbol-12-myristate 13-acetate (PMA) or IL-1. Zubler *et al.*, Immunological Reviews (1987) 99:281; and Zhang *et al.*, J. Immunol. (1990) 144:2955. B-cell activation in this culture system is efficient -- limiting dilution experiments have shown that the majority of human B cells can be activated to proliferate and differentiate into antibody-secreting cells. Wen *et al.*, Eur. J. Immunol. (1987) 17:887.

The mechanism by which these mutant EL-4 cells activate both murine and human B cells has not been elucidated previously. It is, however, clear that cell-cell contact is required for EL4B5-induced B-cell activation. First, B cells do not proliferate in the presence of supernatant from PMA-stimulated EL4B5 cells. Zubler *et al.* (1985) supra. Second, B cells do not proliferate when they are separated from PMA-treated EL4B5 cells by a semipermeable filter membrane. Zhang *et al.*, supra. Antibodies against mouse LFA-1, human LFA-1 or human LFA-3 and antibodies against mouse or human MHC class II molecules do not inhibit EL4B5-induced proliferation of human or murine B cells. Zubler *et al.* (1987) and Zhang *et al.*, supra.

III. The CD40 Antigen, the CD40 Antigen Ligand, and Anti-CD40 Antibodies

The CD40 antigen is a glycoprotein expressed on the cell surface of B cells. During B-cell differentiation, the molecule is first expressed on pre-B cells and then disappears from the cell surface when the B cell becomes a plasma cell. Crosslinking of the CD40 molecules with anti-CD40 antibodies mediates a variety of effects on B cells. The CD40 antigen is known to be related to the human nerve growth factor (NGF) receptor and tumor necrosis factor-alpha (TNF-α) receptor, suggesting that CD40 is a receptor for a ligand with important functions in B-cell activation.

A ligand for CD40 has been identified on the cell surface of activated T cells. Fenslow *et al.*, J. Immunol. (1992) 149:655; Lane *et al.*, Eur. J. Immunol. (1992) 22:2573; Noelle *et al.* Proc. Natl. Acad. Sci. (USA) (1992) 89:6550. cDNA cloning of the CD40 ligand revealed a molecule with characteristics of a type-II transmembrane glycoprotein with homology to TNF-α. Armitage *et al.*, Nature (1992) 357:80 and Spriggs *et al.*, J. Exp. Med. (1992) 176:1543. The extracellular domain of the CD40 ligand contains two arginine residues proximal to the transmembrane region, providing a potential proteolytic cleavage site that could give rise to a soluble form of the ligand. Expression of recombinant CD40 ligand has demonstrated that this molecule can stimulate the proliferation of purified B cells and, in combination with IL-4,

mediate the secretion of IgE. Armitage *et al.* and Spriggs *et al.*, *supra*. It has been reported that abnormalities in the gene for the CD40 ligand, resulting in the absence of a functional molecule on activated T cells, is responsible for the occurrence of X-linked hyper-IgM syndrome, a rare disorder characterized by the inability of these patients to produce normal levels of antibody isotypes other than IgM. Allen *et al.*, Science (1993) 259:990; and Korthäuer *et al.*, Nature (1993) 361:539.

All anti-CD40 antibodies known in the art have a stimulatory effect on human B cells. Cross-linking of the CD40 molecule on the B-cell surface using known anti-CD40 antibodies mediates a variety of effects on B cells. Anti-CD40 monoclonal antibodies (mAbs) can induce intercellular adhesion, proliferation and, in combination with certain cytokines, maturation to antibody secreting cells. For example, known anti-CD40 mAbs have been shown to mimic the effects of T helper cells in B-cell activation. When presented on adherent cells expressing FcγRII, these antibodies induce B-cell proliferation. J. Bancereau *et al.*, Science (1989) 251:70. Moreover, the known anti-CD40 mAbs can replace the T helper signal for secretion of IgM, IgG and IgE in the presence of IL-4. H. Gascan *et al.*, J. Immunol. (1991) 147:8. Furthermore, known anti-CD40 mAbs can prevent programmed cell death (apoptosis) of B cells isolated from lymph nodes.

However, the anti-CD40 antibodies known in the art stimulate B cells but are incapable of inhibiting the B-cell response. Furthermore, no anti-CD40 antibodies are known that are (1) capable of inhibiting the B-cell response and (2) can be used to prevent or treat antibody-mediated disease.

SUMMARY OF THE INVENTION

The current invention is based on the discovery of anti-CD40 antibodies that do not stimulate the growth and differentiation of human B cells. In contrast, these antibodies can inhibit the human B-cell response at relatively low concentrations. Accordingly, these antibodies can be used to prevent or treat diseases or conditions that are mediated by antibodies produced by the human B-cell response. These antibodies also recognize novel epitopes on the CD40 antigen useful in modulating the B-cell response.

Accordingly, the present invention is directed to providing a monoclonal antibody or an antigen binding fragment thereof capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody or fragment to the CD40 antigen prevents the growth or differentiation of the B cell, the humanized monoclonal antibody having

an effective number of exposed amino acid residues in its framework regions that are consistent with the amino acid residues usually found in the corresponding framework regions of a human antibody to provide a reduced immunogenicity in humans. Hereinafter, it is understood that the term "monoclonal antibody," as used in the context of the anti-CD40 monoclonal antibodies of the claimed invention, includes antigen binding fragments thereof.

The present invention is also directed to providing a method for preventing or treating an antibody-mediated disease in a patient, the method comprising administering to a patient in need of such treatment a composition comprising: *(i)* a therapeutically effective amount of a monoclonal antibody (or fragment thereof) that is capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, the antibody having an effective number of exposed amino acid residues in its framework region that are consistent with the amino acid residues usually found in the corresponding framework regions of a human antibody to provide a reduced immunogencity in humans, and *(ii)* a pharmaceutically acceptable excipient.

It is another aspect the present invention is directed to providing a method for preventing or treating an IgE-mediated disease such as an allergy in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a monoclonal antibody (or fragment thereof) that is capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, the antibody being in a pharmaceutically acceptable excipient.

Further, the present invention is directed to providing a method for preventing or treating an antibody-mediated autoimmune disease in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a monoclonal antibody (or a fragment thereof) capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient. Particular autoimmune diseases contemplated for treatment by this method include systemic lupus erythematosus (SLE), primary biliary cirrhosis (PBC), idiopathic thrombocytopenic purpura (ITP), and rheumatoid arthritis.

It is a further object of this invention to provide a CD40 antigen epitope capable of competing with the binding of a CD40 antigen to an anti-CD40 monoclonal antibody wherein the binding of that antibody to a human CD40 antigen located on the surface of a human B cell prevents the growth or differentiation of the B cell.

In preferred embodiments of the above methods, the monoclonal antibody is either 5D12, 3A8 or 3C6. In a more preferred embodiment, the anti-CD40 monoclonal antibody or fragment thereof has been humanized by comparing the amino acid sequence of the monoclonal antibody to the amino acid sequences of several of the most homologous human antibodies and performing site directed mutagenesis by changing the amino acids in the framework regions of the variable regions which are exposed and which do not match up with their human counterparts. Such humanized antibodies are preferred because they are less likely to invoke an immune response in humans. In a particularly preferred embodiment, the anti-CD40 monoclonal antibody is a humanized CDR fragment of an anti-CD40 monoclonal antibody that is capable of binding to the CD40 antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows a schematic representation of the baculoviral transfer vector pAcC8 and the sequence of the multiple cloning site. Figure 1B shows a schematic representation of the generation of Sf9 cells which express human CD40 or B7 antigen.

Figure 2 shows the sequences of polymerase chain reaction primers used in the preparation of coding regions for human CD40 and human B7 antigens. These primers were constructed on the basis of the published complete DNA coding sequences for antigens B7 and CD40.

Figure 3 shows the results of ELISA assays examining the reaction of anti-CD40 monoclonal antibody S2C6 with Sf9 cells expressing CD40 and with Sf9 cells expressing B7.

Figure 4 shows the results of the fluorescent cell staining of EBV-transformed B-cell line ARC cells expressing CD40.

Figure 5A compares the ability of three new (5D12, 3C6 and 3A8) and one old (S2C6) anti-CD40 mAbs to co-stimulate anti-IgM induced human B-cell proliferation. Figure 5B repeats the experiment of Figure 5A in the presence of recombinant interleukin-2 (rIL-2).

Figure 6 shows the ability of three new anti-CD40 mAbs to inhibit human B-cell proliferation induced by costimulation with immobilized anti-IgM and anti-CD40 mAb S2C6.

Figure 7 shows the effect of three new anti-CD40 mAbs on EL4B5-induced human B-cell proliferation.

Figure 8 shows the effect of soluble CD40 (hCD40.hμ) on EL4B5-induced human B-cell proliferation.

Figures 9A and 9B show the effect of one new anti-CD40 mAb 5D12 on human T-cell induced immunoglobulin production of human B cells.

Figure 10 shows the DNA sequence for the murine heavy chain variable (VH_M) region of the 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 10 also labels VH_M at the first amino acid of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2, CDR3) that are positioned therebetween in alternating fashion.

Figure 11 shows the DNA sequence for the murine light chain variable (VL_M) region of the 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 11 also labels VL_M the first amino acid of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2 and CDR3) that are positioned therebetween in alternating fashion.

Figure 12 shows the DNA sequence for the humanized heavy chain variable (VH_z) region of 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 12 also labels VH_z the first amino acid of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2 and CDR3) that are positioned therebetween in alternating fashion.

Figure 13 shows the DNA sequence for the humanized light chain variable (VL_z) region of the 5D12 monoclonal antibody and the amino acid sequence encoded thereby, using conventional single letter designations. Figure 13 also labels VL_z at the first amino acid residue of each of the four framework regions (FR1, FR2, FR3 and FR4) and the three complementarity determining regions (CDR1, CDR2 and CDR3) that are positioned therebetween in alternating fashion.

Figure 14 compares the binding to CD40 expressing cells by murine 5D12 monoclonal antibody, chimeric 5D12 Fab, chimeric VL, chimeric VH, humanized VL, humanized VH, and humanized 5D12 Fab, via FACScan flow cytometer curves, relative to each other and to FITC-conjugate as the control. Figure 14 shows that chimeric 5D12 Fab and humanized 5D12 Fab exhibit the same binding to CD40 expressing cells as does the intact 5D12 monoclonal antibody.

Figure 15 is a comparative plot of mean fluorescent intensity (MFI) versus Fab dilutions (1=16, 1=8, 1=4, 1=2 and 1=1) of chimeric 5D12 Fab (solid rectangle) and humanized 5D12 Fab (open ovals). To obtain the curve points, a CD40 expressing B cells (JY cell line) was incubated with 50 ng of intact 5D12 monoclonal antibody together with the diluted amounts of the expressed chimeric or humanized 5D12 Fab in 100 µl for thirty minutes at 4°C., and the MFI

was measured. The 1=1 dilution, which contained 70 ng chimeric 5D12 Fab or 80 ng humanized 5D12 Fab, was able to inhibit 50% of the intact murine 5D12 monoclonal antibody.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications and applications, cited previously or below are hereby incorporated by reference.

Definitions:

As used herein, the term "antibody" refers to polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof such as F_{ab}, $F_{(ab')2}$, F_v, and other fragments, such as CDR fragments, which retain the antigen binding function of the parent antibody.

As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as F_{ab}, $F_{(ab')2}$, F_v, and others, such as CDR fragments, which retain the antigen binding function of the antibody. Monoclonal antibodies of any mammalian species can be used in this invention. In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

As used herein, the term "humanized monoclonal antibodies" means that at least a portion of the exposed amino acids in the framework regions of the antibody (or fragment), which do not match with the corresponding amino acids in the most homologous human counterparts, are changed, such as by site directed mutagenesis of the DNA encoding the antibody. Because these exposed amino acids are on the surface of the molecule, this technique is called "resurfacing." Moreover, because the amino acids on the surface of the molecule are the ones most likely to give rise to an immune response, this resurfacing decreases the immunogenicity of the monoclonal antibody when administered to a species whose cell line was not used to generate the antibody, such as a human. The term "humanized monoclonal antibody" also includes chimeric antibody wherein the light and heavy variable regions of a monoclonal

antibody generated by a hybridoma from a non-human call line are each attached, via recombinant technology, to one human light chain constant region and at least one heavy chain constant region, respectively. The preparation of such chimeric (*i.e.*, humanized) antibodies are disclosed by references incorporated herein by reference.

As used herein, the term "single chain antibodies" refers to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety which permits preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding to the antigen. Determination and construction of single chain antibodies are described in U.S. Patent No. 4,946,779 to Ladner *et al.*, which is expressly incorporated herein by reference.

The term "CD40 antigen epitope" as used herein refers to molecule which is capable of immunoreactivity with the anti-CD40 monoclonal antibodies of this invention, excluding the CD40 antigen itself. CD40 antigen epitopes may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (*i.e.*, organic compounds which mimic the antibody binding properties of the CD40 antigen), or combinations thereof. Suitable oligopeptide mimics are described, *inter alia*, in PCT application US91/04282.

I. Antibody Preparation

Anti-CD40 monoclonal antibodies 5D12, 3A8 and 3C6 were prepared as described in Example 1 herein. Humanized anti-CD40 monoclonal antibody fragments were prepared as described in Example 8. Other monoclonal antibodies of the invention may be prepared similarly, or as follows.

a) Polyclonal Sera

Polyclonal sera may be prepared by conventional methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, preferably a mouse, rat, rabbit, or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Immunization is generally performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 μg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or

more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vitro* immunization.

Polyclonal antisera are obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (*e.g.*, 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

b) Monoclonal Antibodies

Monoclonal antibodies are prepared using the method of Kohler and Milstein, Nature (1975) 256:495-96, or a modification thereof, as are well known to the art. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) are removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (*e.g.*, hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the desired immunizing cell-surface antigen (and which do not bind to unrelated antigens). The selected mAb-secreting hybridomas are then cultured either *in vitro* (*e.g.*, in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes.

For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a mAb. Further, one may combine various labels for desired effect. For example, mAbs and avidin also require labels in the practice of this invention; thus, one might label a mAb with biotin, and detect its presence with avidin labeled with ^{125}I, or with an anti-biotin mAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

CD40 Antigen Epitopes

The CD40 antigen epitopes of this invention are molecules that are immunoreactive with anti-CD40 monoclonal antibodies whose binding to a human CD40 antigen located on the surface of a human B cell prevents the growth of differentiation of the B cell. That is, such epitopes compete with the binding of said antibodies to the CD40 antigen. Systematic techniques for identifying these epitopes are known in the art, as described by H.M. Geysen in U.S. Patent No. 4,708,871, which is incorporated herein by reference. Typically, these epitopes are short amino acid sequences. These sequences may be embedded in the sequence of longer peptides or proteins, as long as they are accessible.

The epitopes of the invention may be prepared by standard peptide synthesis techniques, such as solid-phase synthesis. Alternatively, the sequences of the invention may be incorporated into larger peptides or proteins by recombinant methods. This is most easily accomplished by preparing a DNA cassette which encodes the sequence of interest, and ligating the cassette into DNA encoding the protein to be modified at the appropriate site. The sequence DNA may be synthesized by standard synthetic techniques, or may be excised from the phage pIII gene using the appropriate restriction enzymes.

Epitopes identified herein may be prepared by simple solid-phase techniques. The minimum binding sequence may be determined systematically for each epitope by standard methods, for example, employing the method described by H.M. Geysen, U.S. Patent No. 4,708,871. Briefly, one may synthesize a set of overlapping oligopeptides derived from the CD40 antigen bound to a solid phase array of pins, with a unique oligopeptide on each pin. The pins are arranged to match the format of a 96-well microtiter plate, permitting one to assay all pins simultaneously, *e.g.*, for binding to an anti-CD40 monoclonal antibody. Using this method, one may readily determine the binding affinity for every possible subset of consecutive amino acids.

Analogs of the invention are also prepared by standard solid-phase methods, and those methods described in PCT application US91/04282.

Formulations and Methods of Administration

The antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat antibody-mediated diseases such as allergies, SLE, PBC, ITP and RA. To accomplish this goal, the antibodies may be formulated using a variety of acceptable excipients known in the art. Typically, the antibodies are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

Before administration to patients, formulants may be added to the antibodies. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di- or polysaccharides, or water soluble glucans. The saccharide or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylellulose, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C_4 to C_8 hydrocarbon having an -OH group and includes galacitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferable between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution. Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred polymers, 4,179,337; 4,495,285; and 4,609,546 which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or aklanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1,000 and 40,000, more preferably between 2,000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di- and triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf *et al.*, 1988 J. Bio. Chem. 263:15064-15070, and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon *et al.*, Cancer Research (1982) 42:4734; Cafiso, Biochem. Biophys. Acta (1981) 649:129; and Szoka, Ann. Rev. Biophys. Eng. (1980) 9:467. Other drug delivery systems are known in the art and are described in *e.g.*, Poznansky *et al.*, DRUG DELIVERY SYSTEMS (R.L. Juliano, Ed., Oxford, N.Y. 1980), pp. 253-315; M.L. Poznansky, Pharm. Revs. (1984) 36:277.

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for

example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

As stated above, the antibodies and compositions of this invention are used to treat human patients to prevent or treat antibody-mediate diseases such as allergies, SLE, PBC, ITP and RA. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are saline, Ringer's solution, dextrose solution, and Hanks' solution. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. A preferred vehicle is 5% dextrose in saline. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that antibodies are given at a dose between 1 μg/kg and 20 mg/kg, more preferably between 20 μg/kg and 10 mg/kg, most preferably between 1 and 7 mg/kg. Preferably, it is given as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose. If so, the antibodies may be infused at a dose between 5 and 20 μg/kg/minute, more preferably between 7 and 15 μg/kg/minute.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Examples

Materials and Methods

Cell Lines

The mutant mouse thymoma EL-4 subclone EL4B5 was a gift of Dr. R.H. Zubler, Hôpital Cantonal Universitaire, Geneva. Mouse 3T6 transfectant cells expressing hybrid molecules of the HR (high responder) allelic form of human FγRIIa were a gift of Dr. P.A.M. Warmerdam, Department of Experimental Immunology, University Hospital Utrecht, Utrecht, The Netherlands. Warmerdam et al., J. Immunol. (1991) 147:1338. Both cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with gentamicin (80 μg/ml) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, Utah). To avoid

possible loss of B Cell activating capacity, every 4 to 8 weeks a new batch of EL4B5 cells was thawed. The cell lines were periodically tested for mycoplasma contamination by the use of a ^{3}H-labelled DNA probe for mycoplasma ribosomal RNA (GenProbe, San Diego,CA) and were free of mycoplasma during the course of the experiments.

Antibodies and hCD40.hμ Fusion Protein

Anti-CD40 mAb 5D12, 3C6 and 3A8 were generated by immunizing mice with insect cells expressing recombinant human CD40 as shown in Example 1. Anti-(B7) mAb B7-24 was generated in a similar way by immunizing with insect cells expressing recombinant human B7. Anti-CD40 mAb S2C6 was a gift of Dr. S. Pauli (University of Stockholm, Sweden). Pauli et al., J. Immunol. (1989) 142:590. Anti-CD40 mAb G28.5 was donated by Dr. J.A. Ledbetter (Oncogen Corporation, Seattle, WA, USA). Clark et al., PNAS (USA) (1986) 83:4494. Control antibodies were: anti-(β-glucocerebrosidase) mAb 8E4 (IgG1), Barneveld et al., Eur. J. Biochem. (1983) 134:585, and myeloma immunoglobulins MOPC-21 (IgG1) and MOPC-141 (IgG2b) (Sigma, St. Louis, MO). All mAb were used as purified antibody preparations. hCD40.hμ fusion protein was a gift of Dr. P. Lane (Basel Institute for Immunology, Basel, Switzerland) and was used as a 5x concentrated supernatant of transfected J558L cells. Lane et al., Eur. J. Immunol. (1992) 22:2573.

Human B Lymphocytes

B lymphocytes were isolated from tonsils obtained from children undergoing tonsillectomies, essentially as described in De Groot *et al.*, Lymphokine Research (1990) 9:321. Briefly, the tissue was dispersed with scalpel blades, phagocytic and NK cells were depleted by treatment with 5 mM L-leucine methyl ester and T cells were removed by one cycle of rosetting with sheep erythrocytes (SRBC) treated with 2-aminoethyl isothiouronium bromide. The purity of the resulting B lyphocyte preparation was checked with indirect immunofluorescent labelling with anti-(CD20) mAb B1 (Coulter Clone, Hialeah, FL) or anti-(CD3) mAb OKT3 (Ortho, Raritan, NJ) and a FITC-conjugated F(ab')$_2$ fragment of rabbit anti-(mouse Ig) (Zymed, San Francisco, CA), and FACS analysis. The B cell preparations contained (mean $\pm$ SD of 6 isolations): 95 $\pm$ 4% CD20-positive cells and 2 $\pm$ 4% CD20-positive cells and 2 $\pm$ 1% CD3-positive cells.

B-Cell Proliferation Assay

B cells (4 x 10^4 per well) were cultured in 200 µl IMDM supplemented with 10% fetal calf serum in flat bottom 96-well microtiter plates. B cells were stimulated by addition of immobilized anti-(IgM) antibodies (Immunobeads; 5 µg/ml; BioRad, Richmond, CA). Where indicated, 100 U/ml recombinant IL-2 was added. Varying concentrations of mAbs were added at the onset of the microcultures and proliferation was assessed at day 3 by measurement of the incorporation of [^{3}H]-thymidine after eighteen hour pulsing.

Banchereau-Like-B-Cell Proliferation Assay

For testing the ability of anti-CD40 mAbs to stimulate B-cell proliferation in a culture system analogous to that described by Banchereau *et al.*, Science (1991) 251:70, mouse 3T6 transfectant cells expressing the HR allelic form of human FcγRII were used. B cells (2 x 10^4 per well) were cultured in flat-bottom microwells in the presence of 1 x 10^4 transfectant cells (irradiated with 5000 Rad) in 200 µl IMDM supplemented with 10% fetal calf serum and 200 U/ml recombinant IL-4. Before addition of the B cells, the 3T6 cells were allowed to adhere to the culture plastic for at least five hours. Anti-CD40 mAbs were added at concentrations varying from 15 mg/ml to 2000 ng/ml and proliferation of B cells were assessed by measurement of thymidine incorporation at day 7, upon eighteen hour pulsing with [^{3}H]-thymidine.

B-Cell Activation Assay with EL4B5 Cells

B cells (1000 per well) were cultured together with irradiated (5000 Rad) EL4B5 cells (5 x 10^4 per well) in flat bottom microtiter plates in 200 µl IMDM supplemented with 10% heat-inactivated fetal calf serum, 5 ng/ml phorbol-12-myristate 13-acetate (Sigma) and 5% human T-cell supernatant. MAbs were added at varying concentrations at the onset of the cultures and thymidine incorporation was assessed at day 6 after 28 hour pulsing with [^{3}H]-thymidine. For the preparation of T-cell supernatant, purified T cells were cultured at a density of 10^6/ml for 36 hours in the presence of 1 µg/ml PHA and 10 ng/ml PMA. Wen *et al. supra*. T-cell supernatant was obtained by centrifugation of the cells and stored at -20°C. The effectiveness of T-cell supernatants in enhancing proliferation of human B cells in EL4B5-B cell cultures was tested and the most effective supernatants were pooled and used in the experiments.

Human T Cell Helper Assay for Antibody Production by Cells

Example 1

Generation of single chain antibody fragments expressing phage from monoclonal antibodies to human CD40 and CD86:

For the generation of a single chain antibody fragment (ScFv) of the anti-CD40 monoclonal antibody 5D12 both the VH and VL region were amplified by PCR, followed by a second assembly PCR to connect both regions. For this purpose 4 primers were designed (SEQ ID NO:1-4). SEQ ID NO:1 contains a HindIII and SfiI restriction site for cloning purposes followed by a degenerated sequence annealing to the 5' VH region of 5D12. SEQ ID NO:2 contains a degenerate sequence for the 3' part of the VH region followed by a sequence encoding a $((Gly)_4Ser)_3$linker and the 5' part of the VL regions. SEQ ID NO:3 is a degenerated primer having homology with the 5' part of the VL region, while the last primer (SEQ ID NO:4) contains a NotI restriction site and anneals to the 3' part of the VL region. Briefly, these primers were used to separately PCR amplify the VH and VL regions of monoclonal antibody 5D12. As template for this PCR reaction we used a plasmid containing the VH or VL regions of 5D12 (VH: SEQ ID NO:5 and VL: SEQ ID NO:6). The cDNA obtained in this PCR step was gel purified and used in an assembly PCR resulting in the linkage of the V region through the $(Gly_4Ser)_3$ linker. Subsequently the obtained single chain 5D12 construct was digested with the restriction enzymes HindIII and NotI, followed by ligation in pGEM-13Zf (Promega Madison USA). The ligation was transformed in DH5α and plated on LB plates. By sequencing of several clones, a correct 5D12 ScFv clone was found (SEQ ID NO:7).

For the generation of ScFv's reactive with human CD86, we used the same primer set as for 5D12. All the steps in the generation of the ScFv of the anti-CD86 monoclonal antibody Fun-1 were performed as described above for the 5D12 ScFv reactive with human CD40. The V regions of Fun-1 (VH: SEQ ID NO:8; VL: SEQ ID NO:9) were used as template to obtain the anti-CD86 ScFv construct (SEQ ID NO:10).

Example 2

Construction of bi-specific diabody molecules capable of binding to human CD40 and human CD86:

Bi-specific bivalent molecules were generated by shortening the flexible linker sequence in the anti-CD40 ScFv and in the anti-CD86 ScFv, from fifteen residues to five (Gly_4Ser) and by cross-pairing the variable heavy and light chain domains from the two single chain Fv fragments with the different antigen recognition. The construction was

incubated for thirty minutes with 10 µl supernatant of transfected cells containing hCD40.hµ diluted in 100 µl Hank's Balanced Salt Solution supplemented with 0.05% sodium azide (4°C). This was followed by incubation with FITC-conjugated $F(ab')_2$ fragments of rabbit anti-(human IgM) (Central Laboratory of the Netherlands, Blood Transfusion Service, Amsterdam, The Netherlands). As a control, cells were incubated with the FITC-conjugate only. For analysis, a FACScan-4 cytofluorometer (Becton-Dickinson) was used. Non-vital cells were excluded from analysis by the use of propidium iodide.

c) **Humanized Monoclonal Antibodies**

The monoclonal antibodies of the present invention, which are capable of blocking the CD40-CD40 ligand interaction, are a useful therapeutic tool in treating autoimmune diseases. However, because many of the monoclonal antibodies of the present invention are directed from a non-human (*e.g.*, murine) cell line, the antibodies may be recognized over time as foreign by the human recipient and an immune response may be mounted which would neutralize subsequent administrations of the monoclonal antibodies. Because the immunogenicity of a foreign protein is largely determined by the nature of the surface, it was decided that the immunogenicity of any monoclonal antibodies of the present invention that were derived from non-human cell lines could be reduced or abolished if an effective number of the exposed (*i.e.*, surface) amino acids in the framework regions of the antibody were replaced with those amino acids usually found in human antibodies.

Techniques for humanizing monoclonal antibodies are well known in the art. Moreover, the techniques for humanizing a monoclonal antibody which are disclosed in the following publications, are expressly incorporated herein by reference:

1) Rodwell, *"Engineering Monoclonal Antibodies,"* Nature, **342**:99-100 (1989);

2) Reichmann, *et al.*, *"Reshaping Human Antibodies For Therapy,"* Nature, **332**:323-327 (1988);

3) Waldman, Thomas, *"Monoclonal Antibodies in Diagnosis and Therapy,"* Science, **252**:1657-1662 (1991);

4) Oi, *et al.*, *"Chimeric Antibodies,"* BioTechniques, **4**(3):214:221 (1986);

5) Liu, *et al.* *"Production Of A Mouse-Human Chimeric Monoclonal Antibody To CD20 With Patent Fc-Dependent Biologic Activity,"* J. Immunol., **139**:3521-3526 (1987);

6) Beidler, *et al.*, *"Cloning and High Level Expression of A Chimeric Antibody With Specificity For Human Carcinoembryonic Antigen,"* J. Immunol., **141**:4053-4060 (1986);

7) Jones, *et al.*, *"Replacing The Complementarity-Determining Regions In A Human Antibody With Those From A Mouse,"* Nature, **321**:522-525 (1986);

8) Wood, *et al.*, *"The Synthesis And In Vivo Assembly of Functional Antibodies In Yeast,"* Nature, **314**:446-449 (1985);

9) U.S. Patent 4,816,567 (Cabilly, *et al.*) *"Recombinant Immunoglobulin Preparations"*;

10) Morrison, *"Transfectomas Provide Novel Chimeric Antibodies,"* Science **229**:1202-1207 (1985);

11) Better, *et al.*, *"Escherichia coli Secretion of an Active Chimeric Antibody Fragment,"* Science, **240**:1041-1043 (1988);

12) Boulianne, G.L., *et al.*, *"Production of Functional Chimaeric Mouse/Human Antibody,"* Nature **312**:643-646 (1984); and

13) Heuberger, *et al.*, *"A Hapten-specific Chimaeric IgE antibody With Human Physiological Effector Function,"* Nature **314**: 268-270 (March, 1985).

For example, to humanize a monoclonal antibody, the variable heavy (VH) chain and the variable light (VL) chain of the monoclonal antibody to be humanized are cloned, sequenced, compared to a series of the most homologous human sequences and then the DNA sequence encoding the monoclonal antibody is modified by site directed mutagenesis. In particular, messenger RNA encoding the monoclonal antibody to be humanized is obtained from the hybridoma producing that antibody. To obtain mRNA encoding the monoclonal antibody, the hybridoma cells are washed twice with phosphate buffered saline and lysed with quanidium thiocyanate in the presence of 0.7M 2-mercaptoethanol. The cell lysate is layered on a discontinuous CsCl gradient and centrifuged for about sixteen hours at 26,000 rpm in a Beckman centrifuge having an SW28 rotor. The RNA is recovered by dissolving the resulting pellet in diethyl pyrocarbonate-treated H_2O (DEPC-treated H_2O). The RNA is precipitated with ethanol once resuspended in DEPC-treated H_2O, and stored at -70°C.

Amplification of the cDNA encoding the heavy and light chain variable regions of the mAb is accomplished by polymerase chain reaction (PCR) using a set of degenerate primers with

restriction sites for cloning. The typical primer set consists of eight 5' primers for the variable region of the heavy chain (VH) that are combined with one universal 3' primer located in the constant region of the heavy chain (CH), and five 5' primers for the variable region of the light chain (VL) that are combined with one universal 3' primer located in the constant region of the light chain (CL). Optionally, a determination is made that one of the primer sets is best suited for the amplification of the VH and VL regions. Thereafter, multiple PCR runs are performed using a low cycle number to avoid incorporation of PCR mistakes. The PCR products obtained after about 24 amplification cycles are subcloned in the polylinker of a sequencing vector. Several (*e.g.*, six) independent clones are analyzed for both VH and VL. This results in a consensus sequence for the cDNA and the deduced protein sequence of the VH and VL regions, respectively of the monoclonal antibody. The deduced protein sequence is overlayed with the location of the framework regions (FR) and the complementarity determining regions (CDR) which form the antigen-binding site of the antibody.

To produce a humanized antibody with the least likelihood of generating an immune response in a human, the deduced protein sequences of the VH and VL regions are used to search different data bases for human antibody sequence with the best homology to the monoclonal antibody (or fragment if only a fragment is to be humanized.) The results are tabulated for the VH and VL regions. Several (*e.g.*, three) of the best matching human antibody sequences are used to determine which of the non-human amino acids in the exposed framework should be changed to a human amino acid in order to obtain a humanized version of the monoclonal antibody.

PCR primers are designed to change the selected exposed residues from non-human to human. Those skilled in the art recognize that the encoding number of mutagenic primers that are designed depends upon the number of mutagenic sites to be introduced into the VH and VL regions. For example, to introduce a total of seven non-adjacent mutagenic sites in both the VH and VL regions, a total of eight primers are designed for the mutagenesis of the VH region, and likewise eight primers are designed for the VL region. cDNA encoding the non-human sequence is used as template for the PCR reactions. Both the humanized variable heavy region (VH_x) and the humanized variable light region (VL_x) region are constructed in three consecutive PCR steps. After the final PCR step, the PCR construct is subcloned in the polylinker of a sequencing vector.

The VH_x and VL_x subclones are transferred using standard techniques from the sequence vectors to expression vectors that already coded part of the human constant heavy (CH_H) region and the complete human constant light (CL_H) region, thereby producing an expression vectors

encoding VH_xCH_H and VL_xCL_H, respectively. The expression vectors coding VH_xCH_H and VL_xCL_H are cotransfected into Sf9 insect cells, whereupon the transfected insect cells secrete the antibody as a humanized monoclonal antibody fragment.

Alternatively, to prepare a chimeric monoclonal antibody from a non-human monoclonal antibody, the variable regions of the original non-human (*e.g.*, mouse) heavy and light chains (VH_M and VL_M) of the monoclonal antibody, as described above were transferred respectively into the expression above that already coded the CH_H and CL_H regions of a human Fab fragment, thereby producing expression vectors encoding VH_MCH_H and VL_MCL_H into Sf9 insect cells, the transfected insect cells secrete the antibody as a human/mouse chimeric monoclonal antibody fragment.

Example 1

Making Monoclonal Antibodies to B7 and CD40

A. PCR Cloning of CD40 and B7

RNA was isolated from a population of EBV-transformed human spleen cells, essentially as described by Chirgwin *et al.*, Biochemistry (1979) 17:5294. In brief, the cells were washed twice with phosphate buffered sale (PBS) and lysed in 5 M guanidinium thiocyanate in the presence of 0.7 M 2-mercaptoethanol. The cell lysate was layered on a discontinuous CsCl gradient (Chirgwin *et al.*) and centrifuged for sixteen hours at 26,000 rpm in a Beckman SW28 rotor. The RNA was recovered by dissolving the pellet in DEPC-treated H_2O. The RNA was precipitated with ethanol once, resuspended in DEPC-treated H_2O, and stored at -70°C.

Total RNA (10 µg/reaction) was converted to cDNA using random hexamer priming in 50 µl reaction buffer containing 500 units MLV-RT (Bethesda Research Laboratories, Bethesda, MD), 5 µM random hexamer (Pharmacia, Piscataway, NJ), 1 mM $MgCl_2$ and 0.1 mg/ml BSA (bovine serum albumin). After incubation at 37°C for one hour, the samples were boiled for three minutes and stored at -70°C. The DNA encoding the CD40 and B7 molecules was generated by PCR using primers which contained sequences having homology to known CD40 and B7 sequence, where the primers also encoded restriction sites useful for cloning (Figure 2). These primers were based on the published cDNA coding sequences for B7 and CD40. Freeman *et al.*, J. Immunol. (1989) 143:1714, and Stamenkovic *et al.*, EMBO J. (1989) 8:1403. All primers start with a C-G clamp at the 5' end followed by a restriction site for cloning (shown in bold, Figure 2). The underlined sequences in the backward primers, for the cloning of the soluble forms of B7 and CD40, represents an epitope recognized by a monoclonal antibody used

for affinity purification. The numbers in brackets represent the location of the primers relative to the published cDNAs for CD40 and B7.

For PCR amplification, 1 µl of cDNA was mixed with 1 µl (10 picomoles) of a forward primer, 1 µl (10 picomoles) of a backward primer, and 47 µl of PCR mix. The PCR mix consisted of 1.25 units Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT), dNTP mix (0.2 mM each), 10 mM tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml BSA. The 50 µl of PCR mixture was overlaid with 70 µl mineral oil and subjected to 25 cycles of amplification in a Perkin-Elmer/Cetus thermocycler (denaturation at 95°C for thirty seconds, primer annealing at 55°C for thirty seconds and extension at 72°C for 1.5 minutes). PCR products were obtained after 25 amplification cycles.

The amplification products were digested with *BglII* and *KpnI* (Figure 1B) and isolated by size-fractionation. Before expression in baculovirus, the DNA sequence of each fragment was confirmed by sequencing analysis to prevent the introduction of PCT-induced mutations. The baculovirus transfer vector pAcC8 was also digested with *BglII* and *KpnI* (Figure 1B).

The amplified fragments were ligated to the linear pAcC8 vector (ratio of insert to vector was 3:1). The ligation products were transformed into bacterial strain DH5α (Gibco/BRL, Gaithersburg MD) and recombinant pAcC8 vectors were selected on the basis of ampicillin resistance. Recombinant plasmids were isolated from bacterial clones (Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratories, 1982; Ausubel *et al.*, Current Protocols in Molecular Biology (Media, PA: John Wiley and Sons)) and the presence of the insert of interest verified using polymerase chain reactions (*see* above). Large scale plasmid preparation was performed by standard procedures (Ausubel *et al.*; Maniatis *et al.*, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratories), 1989).

B. Baculovirus Expression of Human CD40 and B7

Sequences encoding human CD40 and human B7 were recombined into the *Autographa californica* baculovirus (AcNPV) using the transfer vectors pAcCD40 (encoding the full-length CD40 molecule), pAcCD40-ED/Glu (encoding the extracellular domain of CD40), pAcB7 (encoding the full-length B7 molecule) and pCcB7-ED/Glu (encoding the cellular domain of the B7 molecule).

The plasmids were cotransfected with wild-type baculoviral DNA (2-10 pfu) (AcNPV; Summers *et al.*, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture

Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987) into Sf9 (*Spodoptera frugiperda*) cells at a density of 10^6 cells/ml (Summers *et al.*). Recombinant baculovirus-infected Sf9 cells were identified and clonally purified (Summers *et al.*).

For cell surface expression of recombinant proteins, the cells were harvested after forty-eights hours of culture.

C. Sf9 Insect Cell ELISA

Sf9 insect cells infected with recombinant virus were cultured for 48 hours in 24-well plates. After removal of the tissue culture medium, the plates were incubated for 45 minutes at room temperature (RT) with 0.25 ml of antibody in PBS with 1% BSA (PBS-BSA). After three washes with PBS-BSA, the plates were incubated for 35 minutes at RT with 250 µl of a 1/250 dilution of goat anti-(mouse total Ig) immunoglobulins conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) in PBS-BSA. Unbound peroxidase activity was removed by washing five times with PBS-BSA. Bound peroxidase activity was revealed by the addition of an assay mixture prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-tetramethylbenzidine in ethanol to 10 ml with 10 mM sodium acetate, 10 mM EDTA buffer (pH 5.0) and adding 0.03% (v/v) H_2O_2. The reaction was stopped after ten minutes by adding 100 µl of 1 M H_2SO_4.

The above-described ELISA assays performed on live Sf9 cells gave the following results. Figure 3 presents the data for life Sf9 cells infected with pAcB7 and pAcCD40 which were cultured for 48 hours in 24-well plates. The antibodies used in the ELISA to generate Figure 3 were: S2C6 (anti-CD40, open bars) and no primary antibody (hatched bars).

D. Host Animal Immunization

Female BALB/c mice were injected intraperitoneally at day 0 and day 14 with 5 x 10^6 Sf9 cells infected with AcCD40 virus, AcB7 virus or AcCD3 virus (control virus). At day 21, 100 µl of serum was obtained to test for the presence of specific antibodies. After a rest period of at least two weeks, the mice received a final injection with 5 x 10^6 cells infected with AcCD40 or AcB7 virus. Three days after this last injection, the spleen cells were used for cell fusion.

E. Generation of Hybridoma Clones

Splenocytes from immunized BALB/c mice were fused with SP2/0 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by de Boer *et al.*, J.

Immunol. Meth. (1988)113:143. The fused cells were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, MA). The fused cells were then distributed between the wells of 96-well tissue culture plates, so that each well contained 1 growing hybridoma on average.

After 10-14 days, the supernatants of the hybridoma populations were screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants of twelve wells were pooled and used for fluorescent cell staining of EBV-transformed B cells as described for the FACS

Assay above. Subsequently, the supernatants of the positive pools were tested individually. Positive hybridoma cells were cloned three times by limiting dilution in IMDM/FBS containing 0.5 ng/ml hIL-6. Three hybridomas producing anti-CD40 antibodies are labelled 5D12, 3A8 and 3C6. The data is presented in Figure 4, which shows that a soluble form of CD40, but not of B7 can block the binding of the anti-CD40 mAb 5D12 to CD40 expressing EBV-transformed B cells. Figure 4 shows fluorescent cell staining of ARC EBV-transformed B cells with 5D12 is the presence and absence of soluble B7 and soluble CD40. 5D12 and the soluble B7, soluble CD40, or controls were preincubated at RT for 20 minutes before addition to the ARC cells. Figure 4A shows staining with 5D12 (dotted line) or second antibody only (solid line). Figure 4B shows staining with 5D12 alone (dotted line) or preincubated with soluble B7 (solid line). Figure 4C shows staining with 5D12 alone (dotted line) or preincubated with soluble CD40.

Example 2

Costimulation of B-Cell Proliferation Using Anti-CD40 mAbs

Four hybridomas producing monoclonal antibodies against human CD40 were generated in Example 1. These mAbs were shown to bind to a similar proportion of tonsillar B cells as anti-CD40 mAb G28.5 does. de Boer *et al.* J. Immunol. Methods (1992) 152:15. Three of these monoclonal antibodies (5D12, 3A8 and 3C6) which were of the IgG2b subclass, were tested for their ability to deliver activation signals to human B cells in the B-cell proliferation assay described above.

Human tonsillar B cells (4 x 10^4 per well) were cultured in 200 µl in microwells in the presence of anti-IgM coupled to Sepharose beads (5 µ/ml) (Figure 5A) or in the presence of anti-IgM plus rIL-2 (100 U/ml) (Figure 5B), Varying concentrations of the anti-CD40 mAbs S2C6, 5D12, 3C6 or 3A8 were added and [^{3}H]thymidine incorporation was measured at day 3 after 18 hours of pulsing. Data presented in Figure 5A are means derived from experiments with B-cell

preparations from three different donors with duplicate incubations. Data of Figure 5B are means of duplicate incubations from one experiment out of two with comparable results.

None of the novel anti-CD40 mAbs was able to significantly costimulate human B-cell proliferation in the presence of immobilized anti-IgM or in the presence of immobilized anti-IgM and IL-2. In contrast, anti-CD40 mAb S2C6 costimulated human B-cell proliferation in a concentration dependent fashion.

Example 3

Induction of B-Cell Proliferation Using Anti-CD40 mAbs

The mAbs tested in Example 2 were tested for their ability to induce proliferation of human B cells in the Banchereau-like Assay described above, *i.e.*, by presenting the anti-CD40 mAb on adherent cells expressing FcγRII. As antibody presenting cells, mouse 3T6 transfectant cells expressing the HR allelic form of human FcγRII were used. It was observed that anti-CD40 mAb S2C6 together with IL-4 induced substantial proliferation of tonsillar human B cells in this system, as assessed by measurement of [^{3}H]thymidine incorporation. Anti-CD50 mAbs 5D12, 3C6 or 3A8, however, did not induce proliferation of human B cells in this culture system (data not shown).

Example 4

Inhibition of S2C6 Stimulated B-Cell Proliferation Using Anti-CD40 mAbs

The mAbs were also tested for their ability to inhibit the costimulation of human B-cell proliferation by anti-CD40 mAb S2C6 using the B-cell proliferation Assay described above. Human tonsillar B cells (4 x 10^4 per well) were cultured in 200 µl in microwells in the presence of anti-IgM coupled to Sepharose beads (5 µg/ml) and anti-CD40 mAb S2C6 (1.25 µg/ml). Varying concentrations of anti-CD40 mAbs 5D12, 3C6 or 3A8 were added and [^{3}H]thymidine incorporation was assessed after three days. As a control anti-(glucocerebrosidase) mAb 8E4 was added in similar concentrations. Barneveld *et al.* Eur. J. Biochem. (1983) 134:585. Data are means ± S.D. derived from experiments with B cells from two different donors with duplicate incubations.

It was found that each of the anti-CD40 mAbs 5D12, 3A8 and 3C6 could inhibit the costimulation of anti-IgM induced human B-cell proliferation by mAb S2C6 (Figure 6). In contrast, no significant inhibition was seen with equivalent amounts of non-relevant mAb 8E4, directed to β-glucocerebrosidase. Barneveld *et al.*, *supra*. Thus, it as concluded that these anti-

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACCTCCAGC TGACCCAGTC TCCACTCTCC CTGCCTGTCA GTCTTGGAGA TCGAGCCTCC 60

ATCTCTTGCA GATCTAGTCA GAGCCTTGTA AACAGTAATG GAAACACCTA TTTACATTGG 120

TACCTGCAGA AGCCAGGCCA GTCTCCAAAG CTCCTGATCT ACAAAGTTTC CAACCGATTT 180

TCTGGGGTCC CAGACAGGTT CAGTGGCAGT GGATCAGGGA CAGATTTCAC ACTCAAGATT 240

AGCAGAGTGG AGGCTGAGGA TGTGGGAGTT TATTACTGCT CTCAAAGTAC ACATGTTCCG 300

TGGACGTTCG GTGGAGGCAC CAAGCTGGAA ATCAAACGA 339

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 723 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAGGTGCAGC TGCAGGAGTC TGGACCTGGC CTGGTGAAAC CCTCACAGAG CCTGTCCATC 60

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TCAGCTCTCA AATCCAGACT GACCATCAGC AAGGACACCT CGAAGAACCA GGTCTTCTTA 240

AAAATGAACA GTCTGCGAGC TGAGGACACA GCCATGTACT ACTGTGTCAG AACCGATGGG 300

GACTACTGGG GCCAAGGGAC CACGGTCACC GTCTCCTCAG GTGGAGGCGG TTCAGGCGGA 360

GGTGGCTCTG GCGGTGGCGG ATCGGACATC GAGCTCACTC AGTCTCCACT CTCCCTGCCT 420

GTCAGTCTTG GAGATCGAGC CTCCATCTCT TGCAGATCTA GTCAGAGCCT TGTAAACAGT 480

AATGGAAACA CCTATTTACA TTGGTACCTG CAGAAGCCAG GCCAGTCTCC AAAGCTCCTG 540

ATCTACAAAG TTTCCAACCG ATTTTCTGGG GTCCCAGACA GGTTCAGTGG CAGTGGATCA 600

GGGACAGATT TCACACTCAA GATTAGCAGA GTGGAGGCTG AGGATGTGGG AGTTTATTAC 660

TGCTCTCAAA GTACACATGT TCCGTGGACG TTCGGTGGAG GCACCAAGCT GGAAATAAAA 720

CGG 723

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 375 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAGGTCCAAC TGCAGCAGTC TGGACCTGAG CTGGAGAAGC CTGGCGCTTC AGTGAAGATA 60

TCCTGCAAGG CTTCTGGTTA CTCATTCACT GACTACAACA TGAACTGGGT GAAGCAGAGC 120

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AATCAGAAGT TCAAGGGCAA GGCCACATTG ACTGTAGACA AATCCTCCAG CACAGCCTAC 240

ATGCAGCTCA ACAGCCTGAC ATCTGAAGAC TCTGCAGTCT ATTTCTGTGC AAGATGGGAC 300

TATAGGTACG ACGACGGGAG GGCTTACTAT GTTATGGACT TCTGGGGTCA AGGAACCTCA 360

GTCACCGTCT CCTCA 375

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAGCTCCAGA TGACCCAGTC TCCATCATCT CTGGCTGCGT CTGCAGGAGA AAAGGTCACT 60

ATGAGCTGTA AGTCCAGTCA AAGTGTTTTA TACAGTTCAA ATCAGAAGAA CTACTTGGCC 120

TGGTACCAGC AGAAACCAGG GCAGTCTCCT AAACTGCTGA TCTACTGGGC ATCCACTAGG 180

GAATCTGGTG TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACACATTT TACTCTGACC 240

GTCAGCAGTG TGCAAGCTGA AGACCTGGCA GTTTATTACT GTCATCAATA CCTCTACTCG 300

TGGACGTTCG GTGGAGGCAC CAACCTGGAA ATCAAACGG 339

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 759 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAGGTCCAAC TGCAGCAGTC TGGACCTGAG CTGGAGAAGC CTGGCGCTTC AGTGAAGATA 60

TCCTGCAAGG CTTCTGGTTA CTCATTCACT GACTACAACA TGAACTGGGT GAAGCAGAGC 120

AATGGAAAGA GCCTTGAGTG GATTGGAAAT ATTGATCCTT ACTATGGTGG TACTAGTTAC 180

AATCAGAAGT TCAAGGGCAA GGCCACATTG ACTGTAGACA AATCCTCCAG CACAGCCTAC 240

ATGCAGCTCA ACAGCCTGAC ATCTGAAGAC TCTGCAGTCT ATTTCTGTGC AAGATGGGAC 300

TATAGGTACG ACGACGGGAG GGCTTACTAT GTTATGGACT TCTGGGGCCA AGGGACCACG 360

GTCACCGTCT CCTCAGGTGG AGGCGGTTCA GGCGGAGGTG GCTCTGGCGG TGGCGGATCG 420

GACATCGAGC TCACCCAGTC TCCATCATCT CTGGCTGCGT CTGCAGGAGA AAAGGTCACT 480

ATGAGCTGTA AGTCCAGTCA AAGTGTTTTA TACAGTTCAA ATCAGAAGAA CTACTTGGCC 540

TGGTACCAGC AGAAACCAGG GCAGTCTCCT AAACTGCTGA TCTACTGGGC ATCCACTAGG 600

GAATCTGGTG TCCCTGATCG CTTCACAGGC AGTGGATCTG GGACACATTT TACTCTGACC 660

GTCAGCAGTG TGCAAGCTGA AGACCTGGCA GTTTATTACT GTCATCAATA CCTCTACTCG 720

TGGACGTTCG GTGGAGGCAC CAACCTGGAA ATCAAACGG 759

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCTCACAGTG CACAGGTGCA GCTGCAGGAG TCTGG 35

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGTGAGAACA TATGGCGCGC CTTATTACCG TTTGATTTCC AGGTTGGTGC C 51

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 764 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTGAAAAAAT TATTATTCGC AATTCCTTTA GTTGTTCCTT TCTATTCTCA CAGTGCACAG 60

GTGCAGCTGC AGGAGTCTGG ACCTGGCCTG GTGAAACCCT CACAGAGCCT GTCCATCACA 120

TGCACTGTCT CTGGGTTCTC ATTATCCAGA TATAGTGTAT ACTGGGTTCG CCAGCCTCCA 180

GGAAAGGGTC TGGAGTGGCT GGGAATGATG TGGGGTGGTG GATCCACAGA CTATAATTCA 240

GCTCTCAAAT CCAGACTGAC CATCAGCAAG GACACCTCGA AGAACCAGGT CTTCTTAAAA 300

ATGAACAGTC TGCGAGCTGA GGACACAGCC ATGTACTACT GTGTCAGAAC CGATGGGGAC 360

TACTGGGGCC AAGGGACCAC GGTCACCGTC TCCTCAGGTG GAGGCGGTTC AGACATTGAG 420

CTCACCCAGT CTCCATCATC TCTGGCTGCG TCTGCAGGAG AAAAGGTCAC TATGAGCTGT 480

AAGTCCAGTC AAAGTGTTTT ATACAGTTCA AATCAGAAGA ACTACTTGGC CTGGTACCAG 540

CAGAAACCAG GGCAGTCTCC TAAACTGCTG ATCTACTGGG CATCCACTAG GGAATCTGGT 600

GTCCCTGATC GCTTCACAGG CAGTGGATCT GGGACACATT TTACTCTGAC CGTCAGCAGT 660

GTGCAAGCTG AAGACCTGGC AGTTTATTAC TGTCATCAAT ACCTCTACTC GTGGACGTTC 720

GGTGGAGGCA CCAACCTGGA AATCAAACGG TAATAAGGCG CGCC 764

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCGCGAATTC ATGGACATGA GGGTCCCCGC 30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AGATTTGGGC TCAACTTTCT TGTCCAC 27

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GTGGACAAGA AAGTTGAGCC CAAATCT 27

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCGCGAATTC TTAAGCGGCC GCAGATCCGC CGCCACCCGA CCCACCTCCG CCCGAGCCAC 60

CGCCACCTTT ACCCGGAGAC AG 82

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCGCGCGGCC GCAATGCACG TGGCCCAGCT T 31

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCGCGCGGCC GCCTAGTCAG AATCTGGGCA CGGTTC 36

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCGGCGGATC CATGGACATG AGGGTCCCCG C 31

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GCGCGGATCC CTAACACTCT CCCCTGTTGA AGC 33

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 876 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

ATGAAATACC TATTGCCTAC GGCAGCCGCT GGATTGTTAT TACTCGCGGC CCAGCCGGCC 60

ATGGCCCAGG TCCAACTGCA GCAGTCTGGA CCTGAGCTGG AGAAGCCTGG CGCTTCAGTG 120

AAGATATCCT GCAAGGCTTC TGGTTACTCA TTCACTGACT ACAACATGAA CTGGGTGAAG 180

CAGAGCAATG GAAAGAGCCT TGAGTGGATT GGAAATATTG ATCCTTACTA TGGTGGTACT 240

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GCCTACATGC AGCTCAACAG CCTGACATCT GAAGACTCTG CAGTCTATTT CTGTGCAAGA 360

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CTTGTAAACA GTAATGGAAA CACCTATTTA CATTGGTACC TGCAGAAGCC AGGCCAGTCT 600

CCAAAGCTCC TGATCTACAA AGTTTCCAAC CGATTTTCTG GGGTCCCAGA CAGGTTCAGT 660

GGCAGTGGAT CAGGGACAGA TTTCACACTC AAGATTAGCA GAGTGGAGGC TGAGGATGTG 720

GGAGTTTATT ACTGCTCTCA AAGTACACAT GTTCCGTGGA CGTTCGGTGG AGGCACCAAG 780

CTGGAAATAA AACGGGCGGC CGCAGAACAA AAACTCATCT CAGAAGAGGA TCTGAATGGG 840

GCCGCACATC ACCATCATCA CCATTAATAA GAATCC 876

Claims

1. A soluble molecule capable of binding to the human CD40 antigen and to at least the human CD86 antigen, said antigens being located on the surface of human lymphocytes.

2. A soluble binding molecule according to claim 1, which is an antibody containing an antigen-binding site of an antibody to CD40 and an antigen-binding site of an antibody to CD86.

3. An antibody molecule according to claim 2, which is a trispecific diabody capable of binding to CD40 and to both CD80 and CD86, in particular by containing the antigen-binding site of an antibody to CD40 and the antigen-binding site of an antibody which is cross-reactive with CD80 and CD86.

4. An antibody molecule according to claim 2, which is a bispecific diabody capable of binding to human CD40 and to human CD86, in particular by containing the antigen-binding site of an antibody to CD40 and the antigen-binding site of an antibody to CD86.

5. An antibody molecule according to claim 2, which is a trispecific triabody capable of binding to CD40, CD80 and CD86, in particular by containing the antigen-binding site of an antibody to CD40, the antigen-binding site of an antibody to CD80 and the antigen-binding site of an antibody to CD86.

6. A soluble binding molecule according to claim 1 or 3, which is capable of binding to at least CD86 by means of the extracellular domain of human CTLA-4.

7. An antibody according to claim 4 or 5, wherein the antibody to CD86 is the antibody Fun-1.

8. An antibody according to any one of claims 2-7, wherein the antibody to CD40 is an antagonistic antibody to CD40.

9. An antibody according to any one of claims 2-7, wherein the antibody to CD40 is a non-stimulatory antagonistic antibody to CD40.

10. A recombinant vector comprising the nucleotide sequences encoding the binding molecule fragments according to any one of claims 1-9, operably linked to regulating sequences capable of expressing the antibody molecule in a host cell.

11. A host cell stably transformed with the vector according to claim 9.

12. A method of producing a recombinant molecule capable of binding to the human CD40 antigen and to at least the human CD86 antigen, comprising culturing a host cell and isolating the binding molecule from the culture medium.

13. A pharmaceutical composition for induction of T cell tolerance, containing a therapeutically effective amount of the binding molecule according to any one of claims 1-9 and a pharmaceutically acceptable carrier.

14. A method for treating T cell mediated immune responses, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 1 to 9.

15. A method for preventing allograft transplant rejection, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

16. A method for preventing xeno transplant rejection, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

17. A method for the induction of T cell tolerance, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

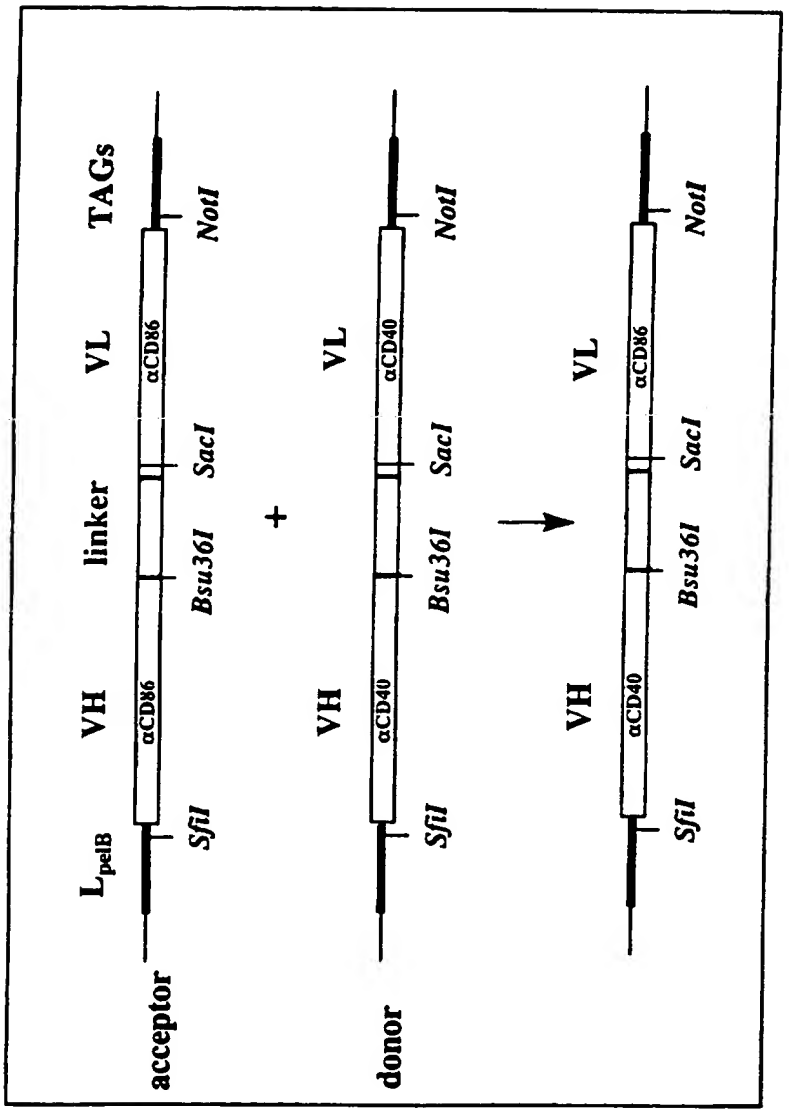
18. A method for the induction of allo-transplant or xeno-transplant tolerance, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

19. A method for preventing or treatment of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and psoriasis, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

20. A method for treating T cell mediated immune responses to gene therapy vectors or vehicles, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

21. A method for treating T cell mediated immune responses to therapeutic molecules, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a pharmaceutical composition according to claim 13.

Figure 1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VH
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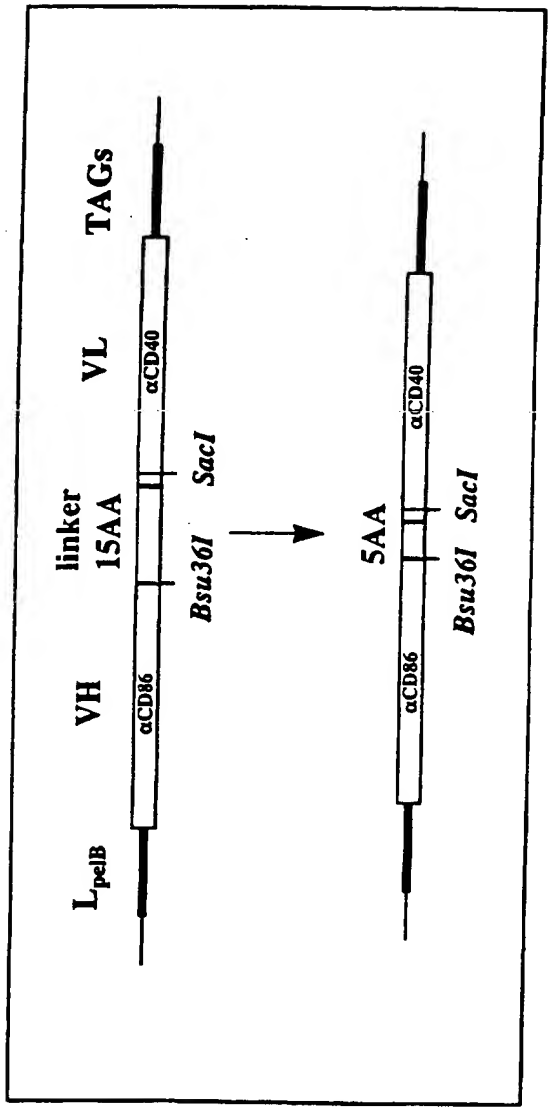
VL
TAGs
αCD86
SfiI
Bsu36I
SacI
NotI
acceptor
+
αCD40
donor


L_{pelB}
VH
αCD86
linker
15AA
Bsu36I
SacI
VL
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TAGs
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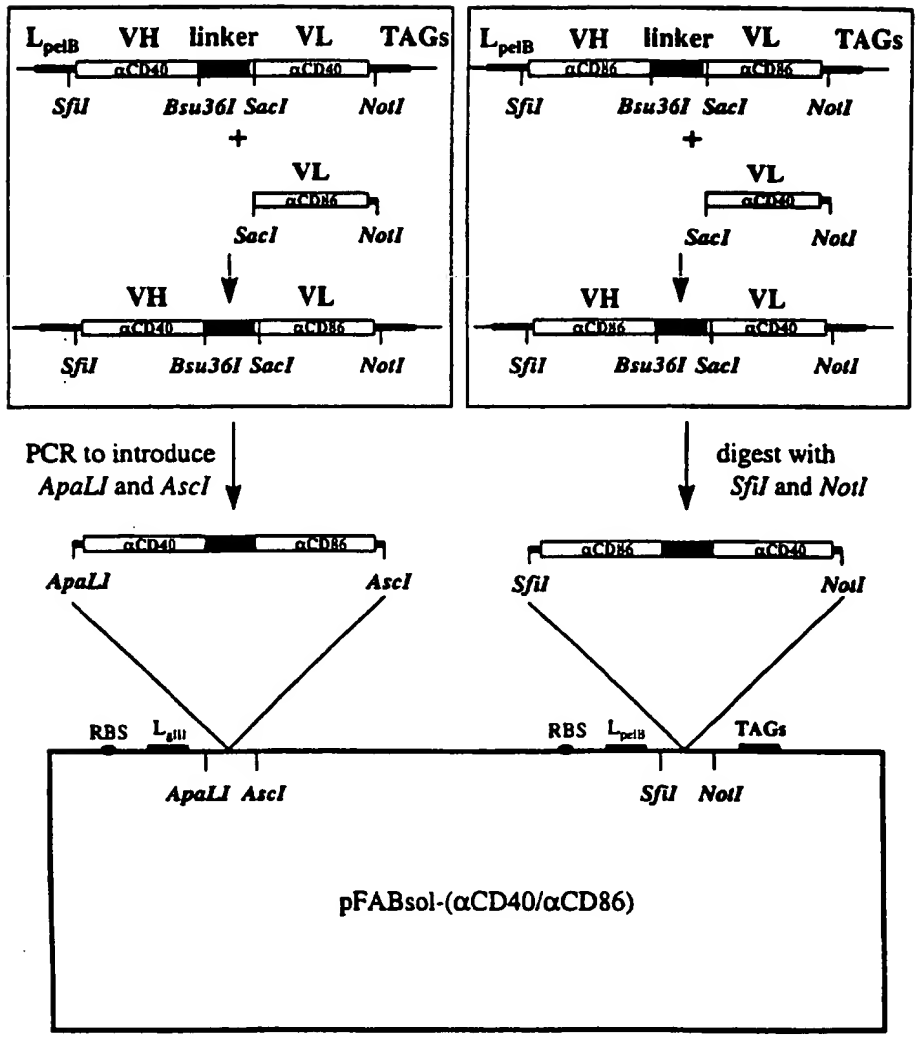
SacI
αCD40

Figure 2

Figure 3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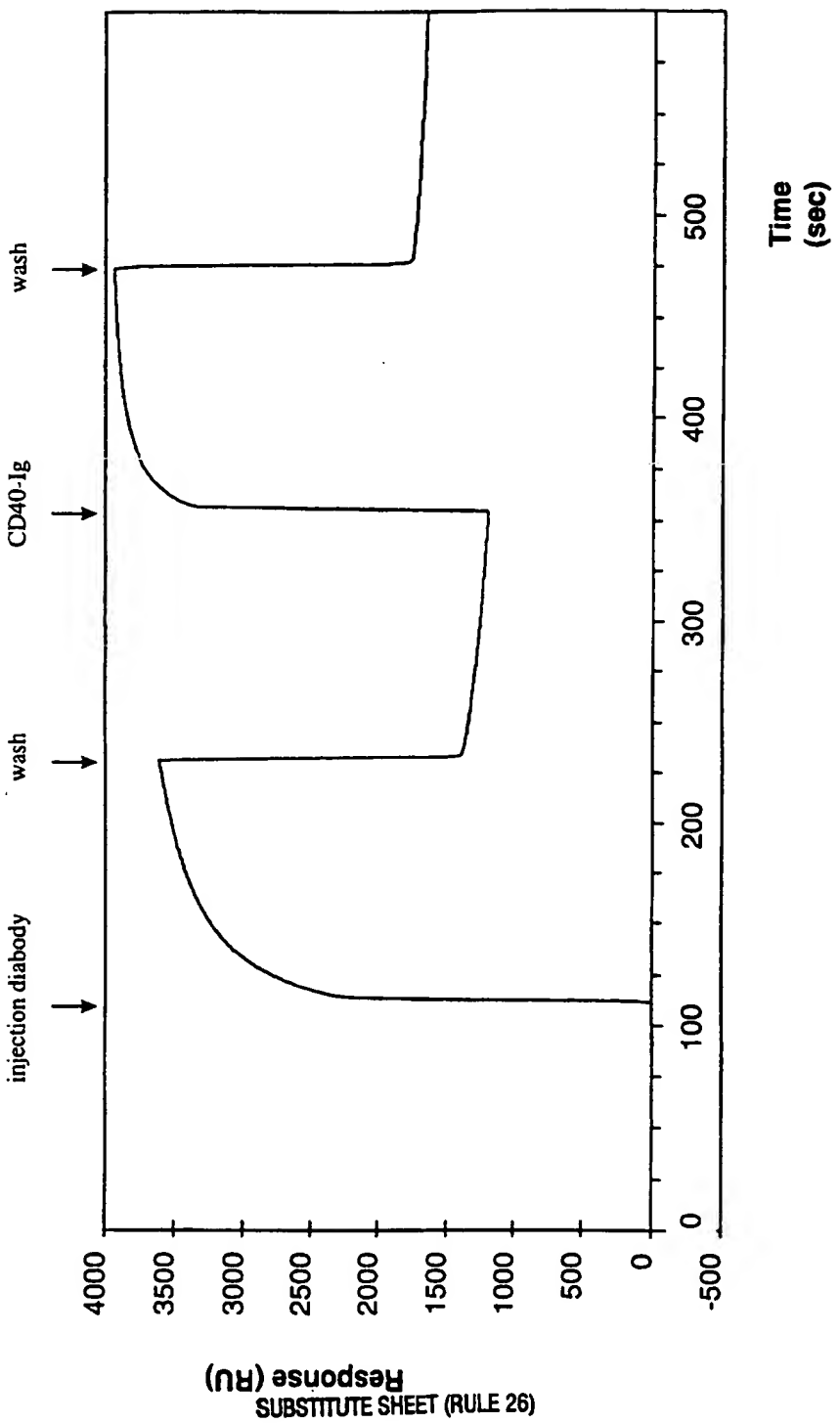
VL
αCD86
SacI NotI
VH VL
αCD40 αCD86
SfiI Bsu36I SacI NotI
L pelB VH linker VL TAGs
αCD86 αCD86
SfiI Bsu36I SacI NotI
+
VL
αCD40
SacI NotI
VH VL
αCD86 αCD40
SfiI Bsu36I SacI NotI
PCR to introduce
ApaLI and AscI
digest with
SfiI and NotI
αCD40 αCD86
ApaLI AscI
αCD86 αCD40
SfiI NotI
RBS L gIII
ApaLI AscI
RBS L pelB
TAGs
SfiI NotI
pFABsol-(αCD40/αCD86)

Figure 4


injection diabody
wash
CD40-Ig
wash
Response (RU)
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3000
2500
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1000
500
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300
400
500
Time (sec)

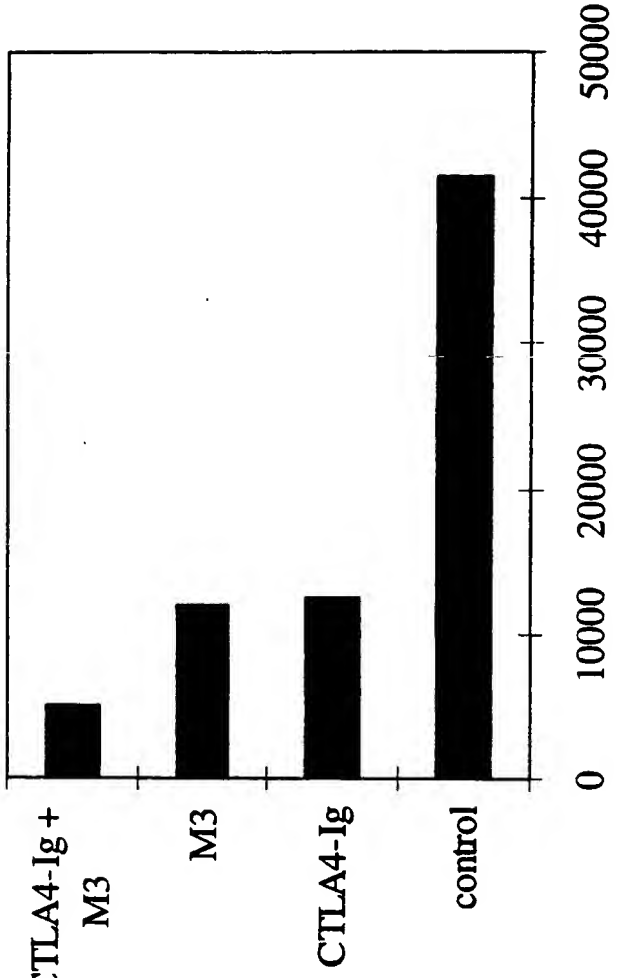

CTLA4-Ig + M3
M3
CTLA4-Ig
control
0
10000
20000
30000
40000
50000
T-cell proliferation in primary MLC (cpm)

Figure 5

Figure 6

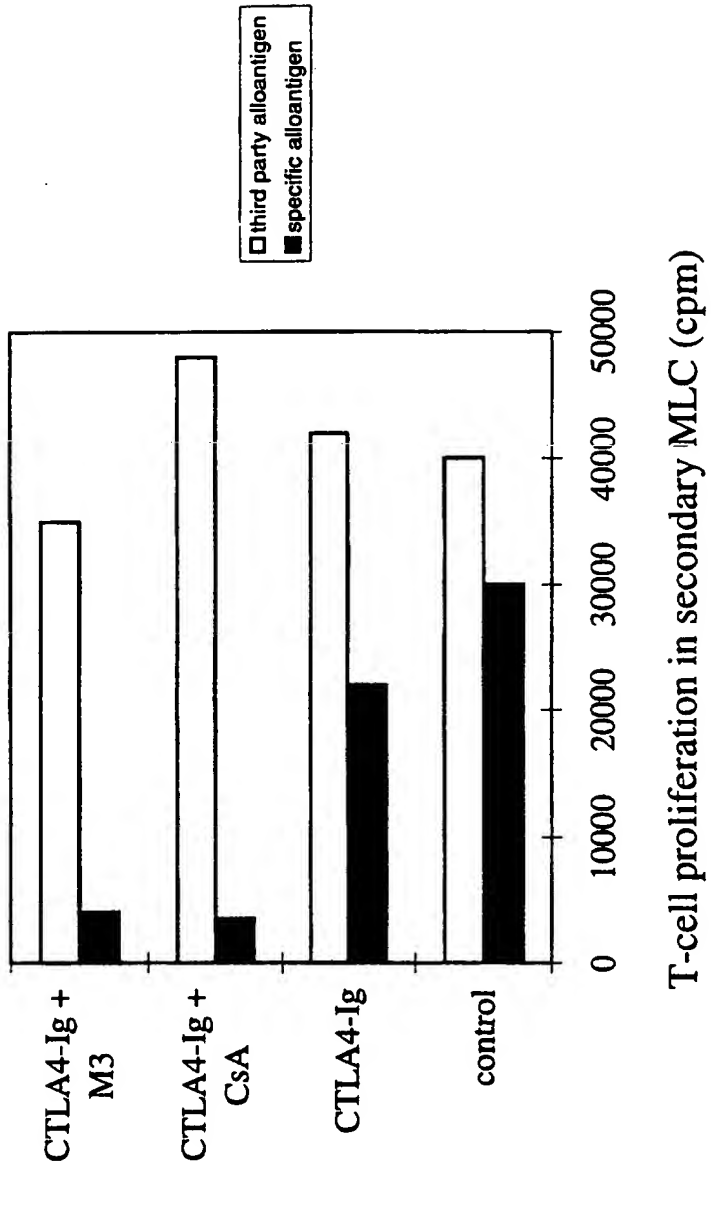
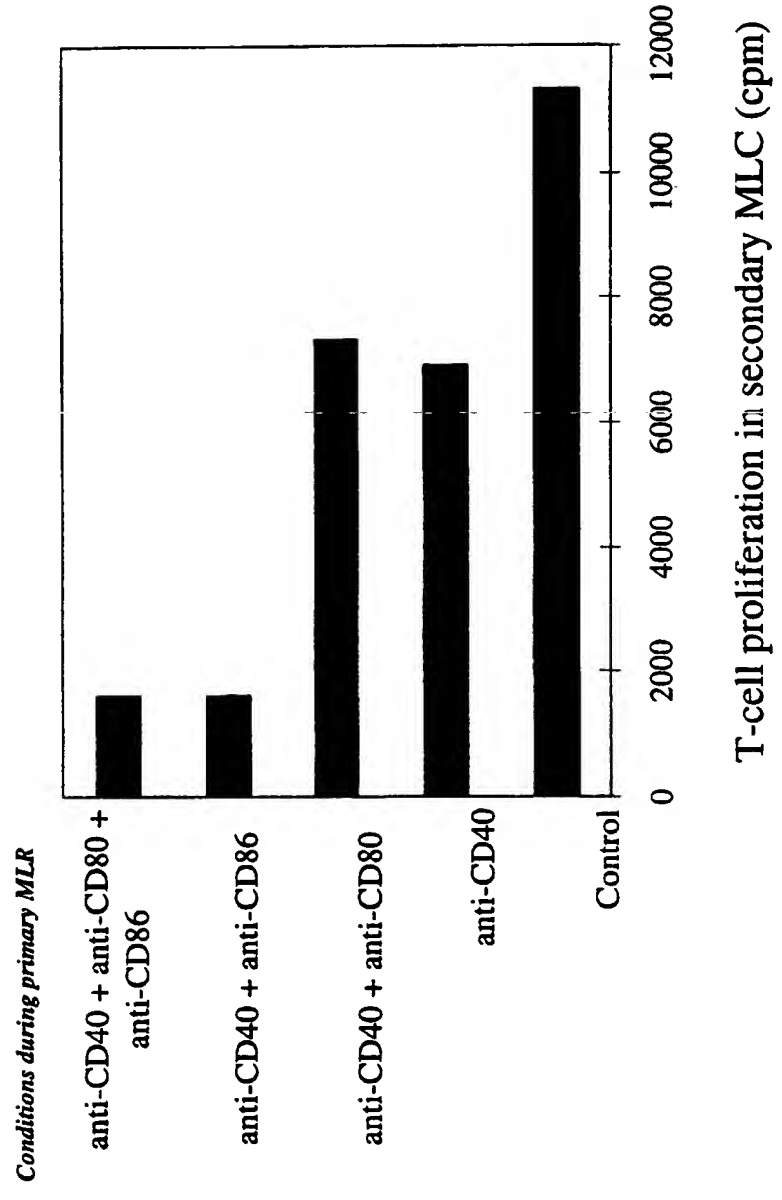

third party alloantigen
specific alloantigen
0
10000
20000
30000
40000
50000
T-cell proliferation in secondary MLC (cpm)
Conditions during primary MLR
CTLA4-Ig + M3
CTLA4-Ig + CsA
CTLA4-Ig
control

Figure 7


Conditions during primary MLR
anti-CD40 + anti-CD80 + anti-CD86
anti-CD40 + anti-CD86
anti-CD40 + anti-CD80
anti-CD40
Control
0
2000
4000
6000
8000
10000
12000
T-cell proliferation in secondary MLC (cpm)

INTERNATIONAL SEARCH REPORT

Intern .nal Application No
PCT/NL 97/00438

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C07K16/46 C07K19/00 C12N5/10 A61K39/395
//C07K1/28,C07K14/705,C12N15/13,C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. LARSEN ET AL.: "Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways." NATURE, vol. 381, no. 6581, 30 May 1996, LONDON, GB, pages 434-438, XP002037035 cited in the application see abstract --- -/--	1,2,6-9, 13-16,18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 October 1997	14. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/NL 97/00438

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	N. GRIGGS ET AL.: "Contribution of CD28/CTLA4/B7 and gp39/CD40 costimulation pathways in clonal expansion and functional acquisition of self reactive T cells." JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT, vol. 0, no. 21 part A, 1995, NEW YORK, NY, USA, page 141 XP002037030 see abstract C2-427 ---	1,2,6-9, 13,14, 17,19
X	N. GRIGGS ET AL.: "The relative contribution of the CD28 and gp39 costimulatory pathways in the clonal expansion and pathogenic acquisition of self-reactive T cells." THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 183, no. 3, 1 March 1996, NEW YORK, NY, USA, pages 801-810, XP002043672 see the whole document ---	1,2,6-9, 13,14, 17,19
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A	M. DE BOER ET AL.: "Generation of monoclonal antibodies to human lymphocyte cell surface antigens using insect cells expressing recombinant proteins." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 152, no. 2, 10 August 1992, AMSTERDAM, NL, pages 15-23, XP002043673 see the whole document ---	1-21
1 A	WO 95 08577 A (MEDICAL RESEARCH COUNCIL) 30 March 1995 see the whole document -----	4,10-12

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/NL 97/00438

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 14-21
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. onal Application No
PCT/NL 97/00438

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